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{Exhibit 29}

**Broker et al., "Electron Microscopic Visualization of
tRNA genes with Ferritin-Avidin: Biotin Labels,"
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Electron microscopic visualization of tRNA genes with ferritin-avidin: biotin labels

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ABSTRACT

A method is described for indirect electron microscopic visualization and mapping of tRNA and other short transcripts hybridized to DNA. This method depends upon the attachment of the electron-dense protein ferritin to the RNA, the binding being mediated by the remarkably strong association of the egg white protein avidin with biotin. Biotin is covalently attached to the 3' end of tRNA using an $\text{NH}_2(\text{CH}_2)_5\text{NH}_2$ bridge. The tRNA-biotin adduct is hybridized to complementary DNA sequences present in a single stranded non-homology loop of a DNA:DNA heteroduplex. Avidin, covalently crosslinked to ferritin, is mixed with the heteroduplex and becomes bound to the hybridized tRNA-biotin. Observation of the DNA:RNA-biotin:avidin-ferritin complex by electron microscopy specifically and accurately reveals the position of the tRNA gene, with a frequency of labeling of approximately 50%.

INTRODUCTION

Electron microscopy has been used to study the organization of genes on chromosomes: (a) by analyzing substitution, deletion and insertion loops in heteroduplex structures prepared between DNA from related genomes (c.f., 1); (b) through cross-annealing identified segments of DNA (such as those present on transducing phages and bacterial F' episomes) (c.f., 2), and (c) by hybridizing purified RNA to complementary sequences in single-stranded (c.f., 3) or double-stranded DNA (c.f., 4). The success of these methods depends upon the existence of sufficiently long duplex regions for reliable discrimination between single and double strands. Methods of indirect visualization of short RNA molecules hybridized to DNA using single strand specific labels such as the T4 gene 32 protein (5) or the *E. coli* binding protein (6) are difficult to apply to very short RNA:DNA hybrid regions. Wu and Davidson

ABBREVIATIONS

NHS - N-hydroxysuccinimide; DMF - dimethylformamide; DMSO - dimethyl sulfoxide; DTT - dithiothreitol; GuHCl - guanidine hydrochloride; NaP - sodium phosphate buffer; NaBH_4 - sodium borohydride.

(7) developed a labeling method using the electron-opaque protein ferritin covalently attached to the tRNA prior to the hybridization step. This technique has proven to be technically demanding.

We have developed an alternative method for attaching ferritin to RNA in an RNA:DNA hybrid. The RNA is covalently attached to the small molecule biotin and is then hybridized to the DNA. The protein avidin is covalently coupled to ferritin. The ferritin-avidin conjugate is then bound to the biotin-RNA:DNA hybrid by means of the strong non-covalent interaction between avidin and biotin.

In the present report we describe the chemical coupling and purification procedures in detail and our studies of the efficiency of electron microscopic mapping, using as a test system, the tRNA^{tyr} gene on the $\phi 80$ psu₃/ $\phi 80$ heteroduplex. The method gives reproducible results with gene labeling efficiencies near 50%. The tRNA genes of HeLa cell mitochondrial DNA have been mapped by this method (8). A further development of the same basic technique is described in the accompanying paper (9).

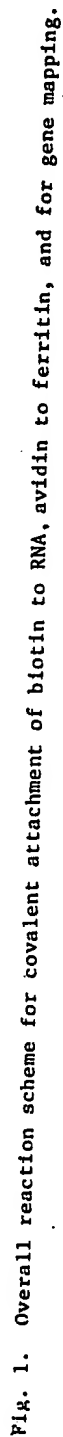
An alternative application of RNA-biotin:avidin technology for mapping genes has been described in a previous publication from this laboratory (10). *Drosophila* ribosomal RNA was coupled to biotin by a different method than the one described here and hybridized *in situ* to salivary gland chromosomes. This preparation was then treated with avidin coupled to polymethacrylate spheres; the hybrids with spheres attached could be visualized in the scanning electron microscope.

RATIONALE

The overall reaction scheme is illustrated in Fig. 1. The 2',3'-cis hydroxyl terminus of tRNA is oxidized by periodate to the dialdehyde and coupled to one of the amino groups of 1,5-diaminopentane by Schiff base formation and subsequent NaBH₄ reduction. Biotin is attached to the remaining amino group of the diamine by acylation with the NHS ester of biotin. The tRNA-biotin conjugate is hybridized to DNA containing the complementary gene sequence. Meanwhile, reactive bromoacetate groups are attached to ferritin and reactive thioacetate groups to avidin by NHS acylation reactions. These groups mediate the crosslinking of ferritin to avidin. The resulting conjugates are used to label the tRNA-biotin:DNA hybrid.

EXPERIMENTAL PROCEDURES

Commercial Materials: A list of purchased materials and their suppliers follows: *E. coli* tRNA, ¹⁴C-cysteine, ¹⁴C-N-ethyl maleimide, GuHCl and sucrose, (Schwarz-Mann); ³H-*E. coli* tRNA, (Miles); avidin and biotin, (Sigma); ¹⁴C-



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biotin, ^3H - NaBH_4 (Amersham-Searle); dithiodiacetic acid, NHS, diaminopentane, (Aldrich); dicyclohexylcarbodiimide (MCB); NaBH_4 (Metal Hydrides, Inc.); Sepharose 2B and 4B (Pharmacia); DEAE-cellulose (BioRad); DMF, sodium iothalamate (Mallinckrodt); ethyl acetate (Baker).

Buffers: The buffers used in some of these experiments are indicated by the following abbreviations: 0.1 M NaCl, 0.01 M NaP, 0.001 M EDTA, pH 7.5 (LXSPE); 1M NaCl, 0.01 M NaP, pH 7.0 (HSB); 1M NaCl, 2M urea, 0.01 M NaP, pH 7.0 (HSUB). All phosphate buffers were prepared from NaH_2PO_4 with the pH adjusted with NaOH.

Specification and Assays: ^3H -tRNA (25,000 daltons MW; 1 mg/ml = 25 A_{260}) was monitored by optical density and by scintillation counting. Avidin (65,000 daltons MW; 1 mg/ml = 1.54 A_{282} (11)) was assayed by optical density and by its irreversible association with ^{14}C -biotin during exhaustive dialysis, sedimentation or chromatography (12, 13). Binding of ^{14}C -biotin is complete within 10 min when a 2- to 5-fold excess is added to avidin. One mg of avidin corresponds to 15.4 nmoles of the tetrameric protein, which bind 62 nmoles (15.1 μg) of biotin (11). Avidin conjugated to Sepharose or to ferritin probably binds less biotin, which makes assays only approximate. Heitzmann and Richards (14) obtained preparations of gluteraldehyde crosslinked ferritin-avidin with yields of biotin binding of approximately 50%. Holo ferritin (900,000 daltons MW; 1 mg/ml = 14.4 A_{280} or 1.54 A_{440} (7, 15)) was assayed by optical density.

NHS-biotin: The NHS ester of biotin was prepared by the method of Becker, Wilchek, and Katchalsky (16). To 12.4 ml of DMF containing 1 g of biotin and 0.475 g of NHS was added 0.85 g of dicyclohexylcarbodiimide. After 15 hr at 25°C, the reaction mixture was chilled to -20°C for 4.5 hr and the dicyclohexylurea precipitate removed by centrifugation. The supernatant was evaporated to dryness in a Buchler vacuum evaporator. The residue was washed with ethanol, dried and then recrystallized from isopropanol. The yield of NHS-biotin was 64%; its m.p. was 198-200°C. NHS- ^{14}C -biotin was synthesized similarly by incubating ^{14}C -biotin (50 μC), biotin (2.4 mg), NHS (1.1 mg) and dicyclohexylcarbodiimide (2.1 mg) in 50 μl DMF for 5.5 hr at 25°C. The resulting solution of ca. 0.2 M ^{14}C -NHS-biotin (Specific activity = 1.4×10^7 cpm/ μmole) was used without further purification. An alternate synthesis of NHS-biotin has been described (17).

NHS-dithiodiacetic acid: To a stirred solution of 1.82 g dithiodiacetic acid (0.01 mole) and 2.3 g NHS (0.02 mole) in 100 ml ethyl acetate was added 4.12 g dicyclohexylcarbodiimide (0.02 mole). The mixture was stirred at room

temperature for 2.5 hr. Dicyclohexylurea was removed by filtration and washed with 40 ml ethyl acetate. The combined filtrates were subjected to rotary evaporation at reduced pressure to yield a dark yellow oil. Several recrystallizations from methanol yielded white needles of the NHS ester with m.p. 131.5 - 135°C.

NHS-bromoacetic acid: The preparation of this ester has been described previously (18).

Synthesis of tRNA-biotin: *E. coli* K12 tRNA at concentrations ranging from 600 µg/ml to 10 mg/ml was incubated for 90 min at 37°C in 2M Tris, pH 8.2 to insure deacylation (19) and was then dialyzed against 0.05 M to 0.1 M sodium acetate, pH 4.7. One tenth volume of 1 M NaIO₄ freshly dissolved in water was added. After oxidation for 1 hr at 20°C in the dark, the tRNA was dialyzed at 4°C in the dark sequentially against 2 changes of 0.05 M sodium acetate, pH 5.1, 0.1 M NaCl, and two changes of 0.3 M sodium borate, pH 9.0 - 9.3 ± 0.1 M NaCl. This solution was made 0.4 M in 1,5-diaminopentane, using a stock of the diamine that had been preadjusted to pH 9.3, and was then incubated for 45-90 min at 20°C in the dark. The resulting Schiff base was reduced with NaBH₄ using unlabeled or ³H-labeled reagent: (i) unlabeled NaBH₄, freshly dissolved in water, was added four times at 30 min intervals, resulting in increments of 0.025 M to 0.1 M BH₄⁻ and incubation was continued for a total of 3 hr at 20°C; (ii) ³H-NaBH₄ (1.28 M and 490 µC/µmole in 1 N NaOH) was added to the Schiff base in 15X molar excess and incubated for 1.5 hr at 0°C, then 2 hr at 20°C. The reduction was then driven to completion by the further addition of unlabeled NaBH₄ as described above. Residual NaBH₄ was quenched by adjustment to pH 5 - 5.5 with 4M sodium acetate, pH 5. The tRNA-amine was dialyzed extensively first against 0.1 M NaCl, 0.01 M- 0.05 M NaP, pH 6.8, 0.001 M EDTA and then against 40% DMF, 0.03 M NaCl, 0.05 M NaP, pH 6.8. NHS-biotin was dissolved in DMF and added to the tRNA-amine (0.5-5 mg/ml) to a final concentration of 20 mM NHS-biotin and about 50% DMF. The reaction was carried out for 12-16 hr at 20°C. Excess NHS-biotin was removed by dialysis against 40% DMF, 0.15 M NaCl, 0.05 M NaP, pH 7.0 and then against 1XSPE. Some preparations of tRNA-biotin were passed through G-25 Sephadex to remove any residual free biotin. The RNA in the excluded volume was precipitated in 70% ethanol for 24 hrs at -20°C and then redissolved in HSB in preparation for chromatography on avidin-Sepharose.

Preparation of Avidin-Sepharose: Avidin was coupled to Sepharose by a procedure similar to those described previously (20, 21, 22). Sepharose 4B (30 µm-200 µm beads) was washed free of sodium azide and was deaerated under

vacuum. 20 ml of Sepharose slurry was suspended in 20 ml water and adjusted to pH 11 with NaOH. 2-4 gm cyanogen bromide, dissolved in 1-2 ml dioxane, was added dropwise over a 3 min period to the Sepharose with gentle mixing. In some preparations, the activation was done at 0°C, in others at 20°C; in either case, the reaction was continued for 10 min while maintaining pH 10.5 - 11.5 with additions of 2 N NaOH. The slurry was poured into a fritted glass filter and washed with about 200 ml 0.1 M sodium carbonate pH 9.0, 0°C. The cake was resuspended in an equal volume of the same buffer. A solution containing 20 mg avidin was added and incubated with the activated Sepharose for 12-24 hr at 4°C. The activated Sepharose was quenched with additional incubation with 0.05 M 2-aminoethanol for 1 hr at 20°C. The avidin-Sepharose was poured into a chromatographic column and successively eluted with 2 vol of HSB, 2 vol of HSUB, 3 vol of 6M GuHCl, pH 2.5, and 5 vol of HSB containing 1 mM EDTA, in which it was stored until use. The effective capacity of the avidin-Sepharose was determined by measuring the amount of ¹⁴C-biotin which could be bound to 1 ml of avidin-Sepharose in HSB and eluted with 6M GuHCl. The procedure just described was found to give 5-15 nmole biotin binding sites per ml of packed bed. An alternative synthesis of avidin-Sepharose has been described recently (23).

To facilitate recovery of tRNA-biotin from avidin-Sepharose, the strongest binding sites on the adsorbent were presaturated with free biotin as follows. Columns of avidin-Sepharose were washed with HSB containing a several fold excess of biotin. They were then washed successively as described above with HSB, HSUB and GuHCl, pH 2.5, to liberate biotin from the weaker binding sites. The columns were regenerated in HSB.

Purification of tRNA-biotin on avidin-Sepharose: tRNA-biotin was purified from residual tRNA and tRNA-amine by selective retention on and elution from a 5 ml column of avidin-Sepharose. The sample was loaded in HSB, and the column was washed with 5 vol of the loading buffer. tRNA without biotin does not bind at this ionic strength. The column was then washed with HSUB to eliminate any tRNA retained by nonspecific hydrophobic interactions. This treatment does not disrupt any but the weakest avidin-biotin interactions. The tRNA-biotin was eluted with 6M GuHCl, pH 2.5, identified by scintillation counting and dialyzed against HSB. The avidin-Sepharose columns were regenerated by washing with 5 vol HSB and could be reused at least several times.

DEAE-cellulose chromatography of tRNA-biotin: tRNA-biotin was chromatographed on DEAE-cellulose columns in 7M urea, 10 mM Tris, pH 8.0, and eluted

with a 0.25 M to 0.5 M NaCl gradient according to the method of Penswick and Holley (24). See legend to Fig. 3 for additional experimental details.

Isolation of Ferritin: Ferritin was purified from horse spleen according to a procedure modified from Granick (25). Three or four horse spleens weighing ca. one kg each were minced in a meat grinder and ferritin was extracted from the residue in 5 l of 80°C water for 10 min. After cooling to 5-10°C in an ice-salt bath, the mixture was filtered through cheesecloth. The filtrate was centrifuged 15 min at 1500 x g. The ferritin was then precipitated by adding solid ammonium sulfate to 35% (w/v). After an overnight incubation at 4°C, the precipitate was collected and redissolved in 2% ammonium sulfate. Insoluble material was removed by centrifugation and the supernatant was made 4% CdSO₄, by adding $\frac{1}{2}$ vol of 20% Cd SO₄, pH 5.8. Ferritin crystals were collected after 3 hr at 4°C, dissolved in 2% ammonium sulfate and the crystallization procedure repeated 4 or 5 times. Ferritin was precipitated twice with 50% ammonium sulphate, dialyzed extensively versus 50 mM NaP, pH 7.0, and sterilized by passage through Millipore HAWP filters (0.45 μ m). Approximately 1 g of ferritin was obtained, which was stored either as an aqueous solution at 2°C or in 50% glycerol, 25 mM NaP, pH 7.0, at -20°C.

Synthesis and purification of ferritin-avidin conjugates: Ferritin (20 mg/ml) in 0.3 M potassium borate, pH 9.3, was bromoacetylated by gentle mixing with about 0.06 vol of a 10 mg/ml solution of the NHS ester of bromoacetic acid in DMSO. After reaction for 1 hr at room temperature, the sample was dialyzed against 1XSPE. The extent of modification was determined by reaction with ¹⁴C-cysteine and measurement of the nondialyzable radioactivity.

The addition of sulfhydryl groups to avidin was accomplished with the same chemistry as outlined above. To a solution of avidin (2.0 - 2.4 mg/ml) in 0.3 M potassium borate, pH 9.3, NHS-dithiodiacetic acid dissolved in DMF was added to give a final ester concentration of 1-3 mg/ml. After reaction for several hr at room temperature, sulfhydryl groups were liberated by treatment for 20 min at 37°C with DTT at a concentration of 12-18 mg/ml. Excess ester and DTT were removed from the reaction mixture by dialysis against 1XSPE under argon. The number of SH groups/avidin was assayed by determining the nondialyzable binding of ¹⁴C-N-ethylmaleimide.

Ferritin-(CCH₂Br)_n was mixed with avidin-(CCH₂SH)_m in 0.3 M potassium borate buffer, pH 9.3, under argon. The ferritin concentration was 8.2 - 10.4 μ M and the avidin concentration was 21-26 μ M. After 2 hr at room temperature, the coupling reaction was quenched by adding 2-aminoethanol (16 M), pH 9.0, to a final concentration of 0.38 M. The mixture was then

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layered on 5-50% sucrose gradients containing HSB + 1mM EDTA built on a 60% sucrose cushion. Ferritin-avidin conjugates and free ferritin were separated from free avidin by two cycles of velocity sedimentation at 36,000 rpm for 7 hr in SW 50.1 rotor at -2°C (or at 40,000 rpm for 4 hr at 0°C).

Hybridization of tRNA-biotin to DNA - the $\phi 80$ psu_3^- system: Phage stocks were prepared as described previously (7). 1.0×10^{10} phage particles of $\phi 80$ wild type and of $\phi 80$ psu_3^- (with a tRNA^{tyr} gene) (each sufficient to contribute 0.5 μg DNA) were diluted into 20 μl of 0.2 M EDTA, pH 8.5, and incubated for 10 min at 20°C . 40 μl H_2O and 20 μl of 1M NaOH were added and incubation was continued for 10 min. The solution was neutralized with 30 μl of 2.5 M Tris HCl, pH 3.5. *E. coli* tRNA-biotin (3-50 μg) was added and the solution was dialyzed versus 40% formamide, 0.3 M NaCl, 0.1 M Tris, 0.001 M EDTA, pH 8.0, for 30-60 min at 40°C . After hybridization, excess tRNA-biotin was removed from the mixture by passage over a 3 ml Sepharose 2B column equilibrated with 1XSPE. The excluded volume was collected and concentrated under vacuum 5- to 7-fold to 50-75 μl .

Labeling DNA:DNA:tRNA-biotin hybrids with ferritin-avidin: Nearly equal volumes of ferritin-avidin and the hybrids were mixed to give a ferritin concentration of 0.1-1 mg/ml (about 10^{-7} - 10^{-6}M), the equivalent of a 1000-10,000-fold excess over the hybridized tRNA-biotin. To allow conjugation, samples were incubated for at least 16 hr at 20°C . Excess ferritin-avidin was removed by centrifuging the mixture through a 5.4 ml gradient of sodium iothalamate ($\rho = 1.2-1.4$), buffered with 0.1 M Tris, 10 mM EDTA, pH 8.0, for at least 8 hr at 35,000 rpm at 15°C in an SW 50.1 rotor. Since the density of DNA in sodium iothalamate is 1.14 (26), while that of ferritin is estimated to be 1.6 - 1.8 (15), ferritin-labeled hybrids can be separated from excess ferritin-avidin. The DNA-containing fractions (0.2 - 0.6 ml from the top of the gradient) were collected manually from the top, dialyzed against 0.8 M NaCl, 0.1 M Tris, 0.01 M EDTA, pH 8.5, and then against 0.2 M Tris, 0.02 M EDTA, pH 8.5. In some cases the samples were concentrated 3- 4-fold in a vacuum dessicator and redialyzed against 0.2 M Tris, 0.02 M EDTA, pH 8.5, in preparation for electron microscopy.

Electron microscopy: The electron microscopic procedures used here are described in more detail in Davis *et al.* (1). The spreading solution contained 50% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5, and 50 $\mu\text{g}/\text{ml}$ cytochrome-c. Depending on the fraction taken from the iothalamate gradient and depending on the experiment, the final DNA concentration ranged from 0.01 - 0.25 $\mu\text{g}/\text{ml}$. The hypophase consisted of 15% formamide, 0.01 M Tris, 0.001 M

EDTA, pH 8.5. The DNA was picked up on parlodion-coated copper grids, stained with 10^{-4} M uranyl acetate and shadowed with 3 - 3.5 cm of platinum palladium (80:20) wire (0.008 gauge) at an angle of 1:9 radians.

Heteroduplex molecules were examined to determine whether they had ferritin labels at the appropriate location on the $\phi 80$ psu_3 strand based on previous mapping (7). Labeled molecules were photographed on 35 mm film at a magnification of 4620 and traced with a Hewlett-Packard digitizer to confirm the location. The percentage of labeling was calculated as the number of correctly labeled molecules divided by one-half the number of heteroduplexes observed (since in half the heteroduplexes the $\phi 80$ psu_3 strand is not the complement of tRNA^{tyr}).

RESULTS

Synthesis of tRNA-biotin: The synthesis of *E. coli* tRNA-biotin was carried out as described in Experimental Procedures. The oxidation reaction was essentially complete as assayed by nondialyzable binding of ^{14}C -isonicotinic hydrazide. Addition of diaminopentane to form a Schiff base was not quantitative and the yield was highly variable among experiments. In previous studies in which oxidized tRNA was treated with cystamine and the product was reduced with DTT and assayed for SH groups with ^{14}C -N-ethylmaleimide, the yields of tRNA-amine varied from 20-80% (7 and our unpublished observations). Similarly, in these experiments, the yield of tRNA-biotin varied from 20-80% as determined either by using ^{14}C -biotin or by binding the reaction mixtures to avidin-Sepharose columns. Since the acylation of primary amines with NHS esters is known to be very efficient, we believe that the variability in yields of tRNA-biotin results from incomplete formation of the Schiff base and/or of its reduction by NaBH_4 . We show below that the procedure causes little if any degradation of the tRNA.

tRNA-biotin conjugates were purified from unmodified tRNA on avidin-Sepharose columns prewashed to remove uncrosslinked avidin subunits and preloaded with biotin to mask the strong binding sites as described in Experimental Procedures. The recovery of tRNA from these columns is usually about 95%. An example of the elution profile is illustrated in Fig. 2. In the top panel, the first passage of tRNA-biotin is shown. In this particular preparation, 50% of the A_{260} bound to the column in 1M NaCl and was eluted in 6M GuHCl, pH 2.5. The bottom panel of Fig. 2 shows that greater than 95% of this material bound on repassage, indicating that the tRNA was not degraded as a result of exposure to the strongly denaturing elution buffer. Further, the excellent rebinding of the tRNA-biotin conjugates suggests that

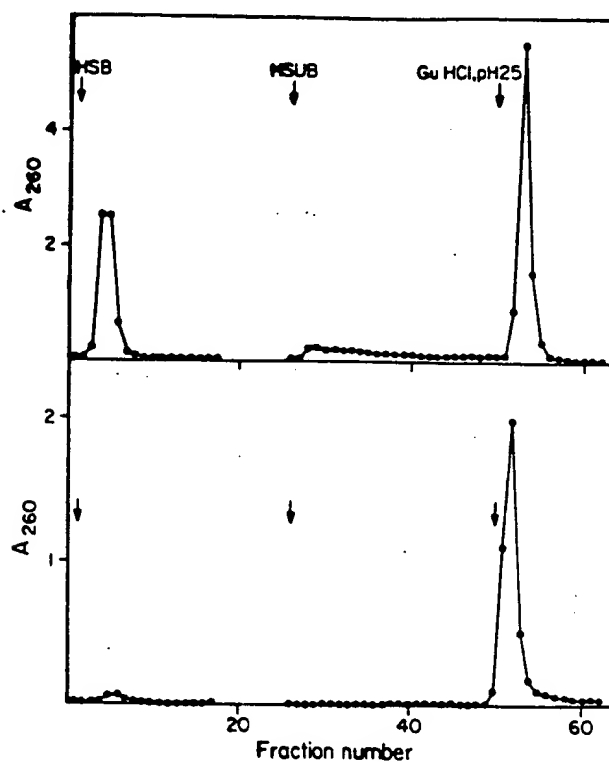


Fig. 2. Affinity chromatography of tRNA-biotin on Avidin-Sepharose. Top: 900 μ g (32 nmoles) of tRNA in HSB was loaded on a 5 ml column of avidin-Sepharose. The total biotin binding capacity of the column was 45 nmoles. Twenty-five ml each of HSB, MSUB and 6 M GuHCl, pH 2.5, were passed through the column. One ml fractions were collected. Bottom: A portion of the material which bound to avidin-Sepharose was rechromatographed on the same column using exactly the same procedure.

it is unlikely that avidin subunits were released during the first pass and became attached to tRNA-biotin.

In one experiment, tRNA-amine was acylated with NHS- 14 C-biotin (Sp. act. = 1.4×10^7 cpm/ μ mole). After purification on avidin-Sepharose, the specific activity of the tRNA-biotin conjugates was 6.2×10^2 cpm/ μ g, indicating that the biotin:tRNA ratio was 1.11:1. Assuming that acylation occurred only at the 3' termini and the tRNA was not degraded, this ratio indicates that the tRNA-biotin preparation is quite pure.

In order to check that the tRNA-biotin was not degraded during the derivatization and purification procedures, it was chromatographed on DEAE-cellulose

in 7M urea, 10 mM Tris, pH 8.0 and eluted with a linear NaCl gradient from 0.25M-0.5M following the procedure of Penswick and Holley (24). According to their results and ours, intact tRNA elutes at ca. 0.38 M NaCl while half size molecules elute at lower ionic strengths (0.31M-0.35M). As shown in Fig. 3, 98% of the tRNA-biotin elutes in a single peak at about 0.38 M indicating that most of the molecules are still full size, or nearly so, and therefore long enough to form stable hybrids. These results also indicate that avidin subunits do not leak from the column and bind to tRNA-biotin. If avidin ($pI = 10.5$) were bound to tRNA-biotin, the elution profile of such complexes which are stable in 7M urea (27) would be different from tRNA alone. Other RNA-biotin samples were analyzed by electrophoresis on 6M urea-15% polyacrylamide gels. No degradation could be detected when the electrophoresis profiles of RNA-biotin and unmodified RNA were compared.

Ferritin-avidin Coupling. Table I shows that, in four separate preparations of bromoacetylated ferritin, between 10 and 21 moles of active bromide were added per mole of ferritin as assayed by the non-dialyzable binding of ^{14}C -cysteine (see footnote a of Table I for details of assay). Attachment of SH groups to avidin was carried out as described in Experimental

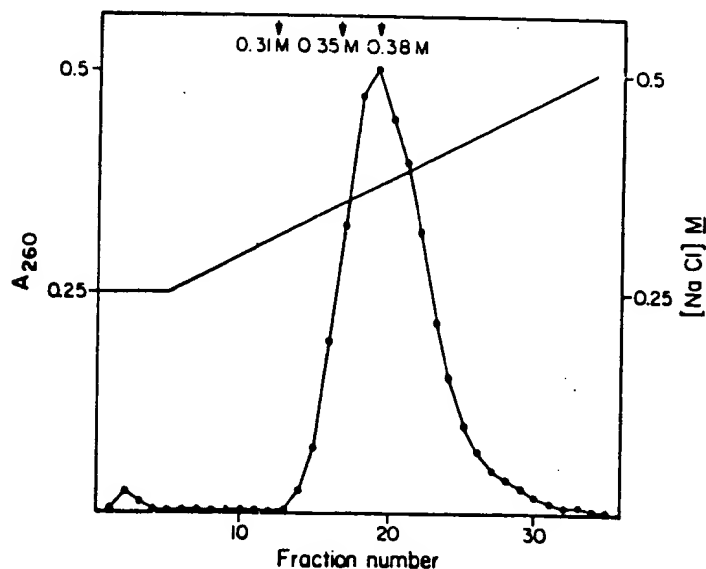


Fig. 3. Chromatography of tRNA-biotin on DEAE-cellulose in 7M urea. 360 μ g of tRNA-biotin was dissolved in 7M urea, 10 mM Tris, pH 8.0, 0.25M NaCl and loaded on a 2.3 ml column of DEAE-cellulose. The column was developed with a 50 ml gradient from 0.25-0.5 M NaCl. 0.7 ml fractions were collected.

Table I
Synthesis of Bromoacetylated Ferritin and Avidin-SH

Experiment Number	Input Molar Ratio		Reaction Time (hours)	± DTT	Molar ratios		
	NHS ester ferritin	NHS ester avidin			(a) ^{14}C cysteine bound ferritin	(b) ^{14}C NEM avidin	(c) ^{14}C biotin avidin
1	100	-	1	-	16.3	-	-
2	120	-	2.5	-	20.8	-	-
2a	10	-	-	-	0.3	-	-
3	105	-	-	-	10.1	-	-
4	95	-	2	-	12.6	-	-
1	-	100	1	+	-	15.0	3.2
2	-	290	1	+	-	10.8	1.9
3	-	100	2	+	-	5.5	2.0
4a	-	100	2	+	-	5.8	4.0
4b	-	0	0	+	-	1.4	3.6
4c	-	0	0	-	-	-	3.7

- (a) Bromoacetylated ferritin was assayed by incubating 10 to 20 picomoles of either modified or unmodified ferritin with 25 nmol of ^{14}C cysteine (Sp. Act. = 15 C/mole) in 50 μl of 0.3 M potassium borate, pH 9.3 under argon for 2 hrs at 25°C. Excess cysteine was removed by dialysis vs. LXSE, 1mM DTT.
- (b) Avidin-SH was assayed by incubating 0.7 to 0.9 nmol of either modified or unmodified avidin with 50 nmol of ^{14}C N-ethylmaleimide (NEM) (Sp. act. = 2.5 C/mole) in 200 μl of LXSE, pH 7.5, for 2 hrs at 25°C. Excess NEM was removed by dialysis versus LXSE.
- (c) Biotin binding was determined by incubating 0.7 to 0.9 nmol of either modified or unmodified avidin with 30 nmol of ^{14}C biotin (Sp. act. = 45 C/mole) in 100 μl of LXSE for 1 hr at 25°C. Excess biotin was removed by dialysis versus LXSE.

Procedures. After liberation of the sulfhydryl groups with DTT, the preparations were dialyzed extensively in oxygen-free buffer and assayed with ^{14}C -N-ethylmaleimide (see footnote b of Table I for details of assay). Although the number of sulfhydryl groups added was somewhat variable among different experiments, all preparations were still capable of binding biotin. In some preparations, no loss of biotin binding was observed (see footnote c, Table I for assay). All of these preparations were able to couple to bromoacetylated ferritin with roughly similar efficiencies.

Modified ferritin and avidin were coupled and purified as described in Experimental Procedures. After the first sucrose gradient sedimentation, 70% of the total biotin binding activity was recovered; the remainder apparently pelleted in insoluble ferritin-avidin aggregates. 65% of the recovered biotin binding activity was associated with the ferritin band in the lower third of the gradient while the remaining 35% was found at the top of the gradient with uncoupled avidin-SH. The fractions containing ferritin were pooled, dialyzed and run on a second identical sucrose gradient and the biotin binding activity of various portions of the gradient determined. 99.9% of the biotin binding activity sedimented with the ferritin band. When

several fractions within the ferritin band were assayed with ^{14}C -biotin, the number of moles of biotin bound per mole of ferritin varied from 2.5 to 4.8, values which correspond to the slower and faster sedimenting ferritin-avidin conjugates, respectively. Electron microscopic examination of these fractions showed that the faster sedimenting material contained more aggregates while the slower sedimenting material was almost entirely ferritin monomers. The monomer fractions were used for the gene labeling experiments. We estimate, both from the recovery of biotin binding activity in these gradients and the number of moles of biotin bound per mole of ferritin, that an average of one to two moles of avidin have been coupled to each mole of ferritin.

The following experiment was done to test whether these ferritin-avidin conjugates could bind tRNA-biotin. Equimolar amounts of tRNA- ^{14}C -biotin (0.65 nmoles, 6900 cpm) and ferritin-avidin (0.65 nmoles ferritin, 2.6 nmoles biotin binding sites) were incubated in 1 x SPE for 24 hours at room temperature. In an identical control reaction, 0.8 nmoles of ^3H tRNA (3.47×10^4 cpm) were incubated with 0.65 nmoles of ferritin-avidin. The reaction mixtures were sedimented 4 hrs at 40,000 rpm at 0°C in the SW 50.1 rotor through a 5-20% sucrose gradient built on a 0.5 ml 60% sucrose cushion containing 2XSPE. As shown in Fig. 4, 100% of the counts in the control reaction (●-●), were recovered and sedimented near the top of the tube while in the tRNA-biotin:avidin-ferritin reaction (x-x), 91.8% of the counts were found associated with the ferritin band in the bottom two fractions of the gradient. Since no absorbance due to tRNA ($\Delta\text{-}\Delta$) was detected at the top of this gradient, we conclude that the ^{14}C counts at the bottom of the gradient represent the binding of at least 90% of the tRNA-biotin in the reaction. Other experiments confirm that our preparations of ferritin-avidin contain little if any ribonuclease activity, since ^3H -tRNA incubated with ferritin-avidin as in the control experiment described above remains full length as assayed in denaturing polyacrylamide gels (data not shown).

Gene Mapping Studies. In order to test the efficiency of labeling DNA: tRNA-biotin hybrids with ferritin-avidin, we have used the heteroduplex formed between the bacteriophage DNAs of $\phi 80$ wild type and $\phi 80$ psu_3^- . $\phi 80$ psu_3^- contains a 3.2 kb sequence of *E. coli* DNA carrying one gene for tyrosine tRNA. The position of this gene in the $\phi 80$ wild type/ $\phi 80$ psu_3^- heteroduplex was mapped in previous studies (7). The hybridization conditions and methods for purifying the hybrids are described in detail in Experimental Procedures. Fig. 5a is an electron micrograph of a heteroduplex labeled with ferritin

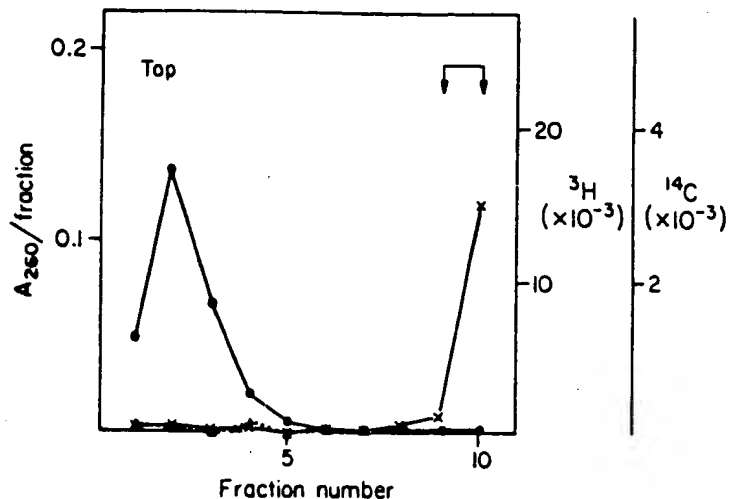


Fig. 4. 5-20% Sucrose gradient sedimentation of reaction mixtures containing either ^3H -tRNA + ferritin-avidin (●-●) or tRNA- ^{14}C -biotin + ferritin-avidin (x-x). The A_{260} due to tRNA-biotin is also indicated (Δ-Δ). Ferritin sedimented to the bottom of the gradient as indicated by the arrow. Note that the concentration of tRNA at the bottom of the gradient cannot be measured by A_{260} because of the large absorbance by ferritin at this wave length.

in the proper position. A histogram of the distribution of labels is presented in Fig. 5b. In this experiment, 106 heteroduplexes were scored for labels. Thirty three heteroduplexes contained ferritin bound to the tRNA gene which is located 1200 nucleotides to the right of the substitution junction at the λ att site. One ferritin was judged to be attached non-specifically. Four ferritins were bound at a position 200 nucleotides to the right of the main peak and, therefore, were not attached to the tRNA^{tyr} gene. This non-random distribution may or may not reflect some weak interaction between the tRNA and DNA at this point.

Several labeling experiments were performed by three different investigators. The combined data are listed in Table II. In these experiments, the hybridizations were carried out over a 25-fold range in rot (rot = (RNA concentration in nucleotide/moles/l) × (time in sec.)). O. Uhlenbeck and his co-workers (personal communication) have determined that the $\text{rot}_{1/2}$ for a pure tRNA under the same conditions is 3×10^{-4} . The lowest rot used based on total tRNA concentration was 0.32, or a rot of 0.016 for tRNA^{tyr}, if this species constitutes about 1/20th of the total, and thus 50 times greater than the required $\text{rot}_{1/2}$. It is clear that there is no correlation between the rot achieved during

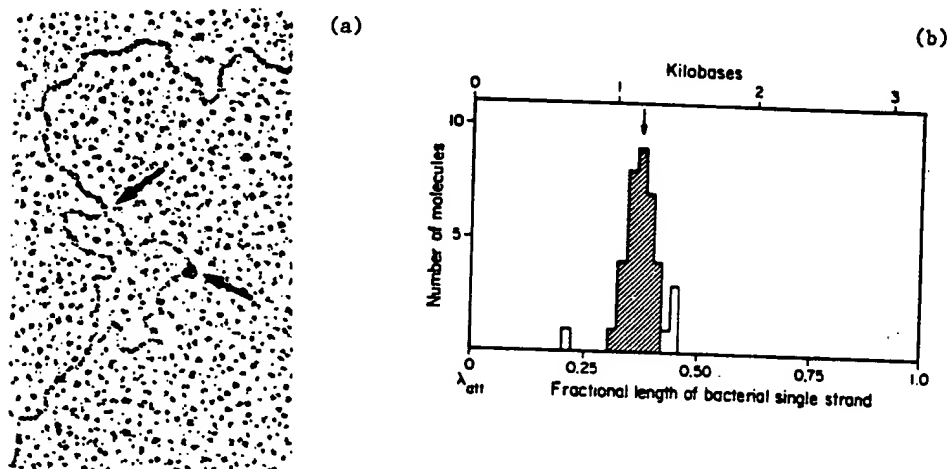


Fig. 5. (a) Electron micrograph of $\phi 80$ wild type/ $\phi 80$ psu_3^- heteroduplex. The arrows indicate the positions of the ferritin label and the ϕatt site. (b) Histogram of distribution of ferritin labels on the 3.2 kb bacterial segment of $\phi 80$ psu_3^- , measured to the right from the att junction near the center of the molecule (7). The att site and the fork at the other end of the substitution loop are 23.8 and 19.3 kb from the left and right ends of the heteroduplex, respectively (5); therefore, they are readily distinguished.

Table II
Efficiency of Gene Labeling

Experiment Number	(a) $cRNA^{tyr}$ rot	Ferritin-avidin $\mu g/ml$	per gene	Labeling time	Number Heteroduplexes	Number genes labeled	(b) % genes labeled
1	1.6×10^{-2}	259	9400	17	42	9	22
2	3.4×10^{-2}	1000	5300	18	25	6	48
3	10×10^{-2}	73	4900	16	106	33	62
4a	42×10^{-2}	16	140	8	25	0	0
4b	42×10^{-2}	9.0	790	8	25	0	0
4c	42×10^{-2}	161	14000	70	25	5	40
4d	42×10^{-2}	211	18500	70	27	8	59

(a) The concentration $cRNA^{tyr}$ is assumed to be equal to 1/20 of the mass of cRNA.

(b) % genes labeled = $\frac{\text{number of labels}}{(4)(\text{number of heteroduplexes})}$

the hybridization and the labeling efficiency. The concentration of ferritin-avidin and the ferritin-avidin:gene ratio were also varied. Only in experiments 4a and 4b (Table II) in which both the ferritin-avidin concentration and ferritin-avidin/gene ratio were low was no labeling observed. In the

other experiments the ferritin concentrations were considerably higher. However, aside from this observation, no clear relationship exists between the gene labeling efficiency and either the concentration or molar excess of ferritin-avidin in the labeling reaction.

Discussion and Experimental Precautions. Several experimental precautions should be observed to maximize the efficiency of this gene labeling procedure. Also some potential side reactions may influence the success of the technique.

Preparations of tRNA may be depleted in certain labile species such as tRNA^{trp} and should be tested for amino acid acceptance by the species to be mapped. Obviously, the existence of isoacceptors reduces the value of this assay unless tRNA samples are fractionated. tRNA preparations may be contaminated with 5S and other small RNAs (and vice versa). Usually these possibilities can be tested by appropriate controls.

tRNA is treated with 2M Tris buffer at pH 8.2 to deacylate any residual amino acids (19); thereafter amine buffers must be absent until the chemical linkage of biotin to the RNA is complete. Excess small molecule reagents such as Tris HCl, NaIO₄, alkane diamines, and NHS-biotin are preferably removed by dialysis or by gel filtration. (Ethanol precipitation of the tRNA does not eliminate all traces of these reagents.) To reduce the possibility of side reactions, solvents and pH are not altered until the reagents are removed. Oxidized tRNA is particularly sensitive to amine-catalyzed beta-elimination of the 3'-terminal nucleotide (28).

Reactions and incubations should be performed in the dark. For instance, periodate-oxidized tRNA dialdehyde is sensitive to photooxidation to the carboxylic acid. The alkane diamines appear to be light-sensitive and should be stored in the dark. The tryptophan residues at the active site of avidin are sensitive to photooxidation in the presence of Fe⁺³ (12), which is a perpetual contaminant once avidin and ferritin have been mixed.

The formation of the Schiff base between oxidized tRNA and the diamine must be driven by as large an excess of amine as is practicable. Diamines shorter than C5 do not provide a linker arm long enough to allow biotin to extend sufficiently far from the macromolecule carrier surface to penetrate the avidin binding sites (29). Diamines longer than about C7 are not sufficiently soluble in water to allow the Schiff base formation to be driven by the necessary concentration excess. Borohydride reduction of the Schiff base presents the opportunity for introduction of a 3'-terminal tritium label from ³H-NaBH₄, allowing the RNA to be traced subsequently during

chromatography and the stability of tRNA-biotin:avidin-ferritin conjugates to be measured.

Several side reactions may cleave certain tRNA species during the modification and purification steps. In particular, the NaBH_4 reduction step modifies 7-methylguanosine and dihydrouridine residues so that adjacent phosphodiester linkages are sensitive to cleavage at low pH (30, 31). *E. coli* tRNA^{tyr} contains a 7-methylguanosine at position 17 from the 5' end. Cleavage at this point would leave a fragment of tRNA extending 68 nucleotides from the biotin-labeled 3' end. This fragment is of sufficient length to chromatograph and hybridize like intact tRNA in the tests described above. Thus, we do not have a critical test to determine whether the present procedure causes significant cleavage at 7-methylguanosine residues. Periodate oxidation probably converts thiouridine to uridine which is not harmful (32). Whether or not the reduction by NaBH_4 of dihydrouridine (32) is deleterious for the present mapping procedure is not known.

Avidin, and particularly avidin-biotin complexes are extraordinarily stable and can withstand heat, pH extremes (2 - 12), urea and 50% formamide (12, 27, 33). The avidin subunits can be separated and the complexes can be denatured in the presence of 6M GuHCl, pH 2.5 or lower (36, 22). Subunit renaturation and reassociation and avidin-biotin binding are freely reversible upon removal of the GuHCl as long as thiol reagents (e.g., DTT) are not present during the denaturation of the avidin (35).

Avidin-biotin complexes are far more stable to subunit dissociation and to denaturation than is free avidin (35). Thus, before exposure to biotin freshly made avidin-Sepharose must be washed with GuHCl, pH 2.5, to remove avidin subunits which were not covalently cross-linked to the matrix. Avidin subunits retain a substantial affinity for biotin (37, 22). Avidin and avidin subunits have a spectrum of biotin affinity constants and it is impractical to reverse the tightest binding (22). Therefore, Sepharose-avidin subunit columns should be exposed first to free biotin to saturate all sites and then eluted with GuHCl, pH 2.5, to free all but the strongest biotin binding sites. These pretreatments will allow good recoveries of tRNA-biotin from the columns and will eliminate any uncrosslinked avidin subunits that might otherwise contaminate the tRNA-biotin upon subsequent elution. Avidin-Sepharose columns are run in the presence of 1 M NaCl at all times to minimize electrostatic interactions between nucleic acids and the very basic avidin.

Ferritin is a somewhat unstable protein and should be freshly recrystal-

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lized before use. It does not withstand freezing or exposure to high salt (e.g., 6 M CsCl). We have noticed that it tends to denature upon prolonged exposure to formamide. Extensive treatment with EDTA may cause substantial release of Fe^{+3} , which may adversely affect avidin (12), nucleic acids and R-SH + R-Br reactions. The free iron concentration should be minimized by dialysis of ferritin solutions shortly before reaction or conjugation steps. To insure the absence of apoferritin and unbound avidin, ferritin-avidin preparations should be sedimented through sucrose gradients immediately prior to use. To prevent pelleting the ferritin-avidin, which is then difficult to resuspend, a cushion of 60% sucrose is used at the bottom of the gradient. The gradient is run at approximately 0 to -2°C to increase its viscosity.

Since labeling of DNA:RNA-biotin should be done at high ferritin-avidin concentrations, it is necessary that ferritin-avidin preparations be absolutely free of unbound avidin. The long incubation opens the possibility for nuclease or protease action and compels care in sterile handling throughout preparation of the sample.

Tolerable background concentrations of ferritin can be achieved if the complexes are prepared for electron microscopy at no more than 1.0 $\mu\text{g}/\text{ml}$ free ferritin-avidin in the hyperphase spreading solutions. Separation of excess ferritin-avidin from hybrids on sodium iothalamate gradients achieves this level of purity.

Ferritin can be confused easily with coarse platinum shadow. Best definition of the label is achieved if a shutter is imposed between the metal filament and the specimen grids during the initial phase of melting and if subsequent evaporation is done just above the melting temperature of the Pt or Pt-Pd. Very light shadowing is required.

FURTHER DISCUSSION

Unlike studies of the distribution of targets such as proteins in surfaces, identification of single features such as genes or proteins on linear chromosomes requires a high labeling efficiency as well as low backgrounds.

The overall efficiency of labeling achieved here is about 40-50%. The percentage of the genes labeled depends upon the $\text{tRNA}^{\text{tyr}}:\text{DNA}$ hybridization and the ferritin-avidin binding efficiencies. It seems plausible that attachment of biotin to tRNA does not significantly alter its rate of hybridization or the stability of the hybrid; therefore, saturation of the gene should be possible at a sufficiently high rot. It is probable that the overall efficiency of this technique is limited by our ability to detect labeled hybrids and by those factors which influence the association of ferritin-

avidin conjugates with biotin-containing hybrids. Such factors include: (1) the stability of ferritin (if iron is lost from ferritin during the course of the experiment, then apoferritin-avidin complexes will be produced which are not visible in the electron microscope); (2) the stability of ferritin-avidin conjugates (traces of free avidin or avidin subunits may react faster with tRNA-biotin hybrids than ferritin-avidin does); (3) the purity of the tRNA-biotin preparation; (4) the stability of the tRNA-biotin linkage; and (5) steric factors which affect the association constants and/or the rates of reaction of ferritin-avidin with biotin-containing hybrids.

We believe that the first three of these factors are not the main cause of the limited labeling efficiency. First, we have used only ferritin-avidin conjugates which have been selected for high density by two cycles of sucrose gradient sedimentation. Second, after the second cycle, we detect less than 0.1% of the total biotin binding activity not associated with ferritin band. Finally, we have shown that tRNA-biotin derivatives are at least 95% pure by repassage through avidin-Sepharose. These derivatives contain an equimolar ratio of biotin to tRNA. The RNA remains full size or nearly full size during modification.

Earlier experiments have shown that the tRNA-amine linkage is stable to our hybridization conditions (7). This result is confirmed with tRNA-biotin by the observation that tRNA-biotin first incubated under hybridization conditions binds ferritin-avidin as extensively as unincubated control samples. Thus, we expect that the tRNA-biotin linkage is stable during our experiments.

Some steric problem which interferes with the avidin reaction might contribute to suboptimal labeling efficiencies. We have found that very high concentrations of ferritin-avidin are required to drive the labeling reaction although much lower concentrations are sufficient to bind unhybridized tRNA-biotin. This observation may be explained by considering the following ideas. First, tRNA-biotin may have a less favorable association with ferritin-avidin than does free biotin. If the linker bridge between biotin and the macromolecule to which it is coupled is not at least 7 or 8 bonds, the equilibrium constant will increase markedly (36, 29). Second, while the chemistry used to modify avidin for subsequent reaction with ferritin does not significantly reduce the number of biotin binding sites, the effect on the equilibrium constant is not known. Finally the concentration of avidin:biotin complex in the spreading solution is very low, about 10^{-11} M. Therefore, the conjugate must be exceptionally stable. With equilibrium binding constants much greater than 10^{-13} M, substantial dissociation would occur in the

time necessary for removal of excess ferritin-avidin or even for spreading the hyperphase during electron microscopic grid preparation. The equilibrium constant for free avidin and biotin at pH 7 in high salt (0.1 M NaCl) is about $10^{-15} - 10^{-13}$ M (13, 22). If the tRNA-biotin:avidin-ferritin association is weaker by several orders of magnitude, perhaps only a small fraction of ferritin-avidin conjugates are capable of forming stable associations under our labeling and spreading conditions.

With this caveat, the avidin-biotin affinity pair satisfies all requirements for speed and stability of association, for availability of materials, for ease of attachment to a wide variety of macromolecules and solid supports, and for convenient assay. All necessary reactions that might affect the integrity of biological materials can be done between pH 5 and 9 and at temperatures no higher than 37°C in aqueous or compatible mixed solvents. The reactions are versatile and strategies can be devised and executed in which either avidin or biotin can be coupled to any of the components which must be conjugated. For example, in addition to gene mapping studies, the avidin:biotin linkage has recently been used to enrich the 18s and 28s ribosomal genes (38, 39) and the 5s ribosomal genes from whole *Drosophila* DNA (9).

In contrast to a method previously described (7), in which ferritin was conjugated directly to tRNA before its hybridization to DNA, the scheme reported here allows the label to be added to established DNA:tRNA-biotin hybrids. In principle, this should allow more efficient nucleic acid hybridization and higher labeling efficiencies. Furthermore, the ferritin label is added after the hybridization in a nondenaturing solvent which preserves ferritin structure. In practice, the maximum efficiencies of both methods are about 60%, but the ease of handling, the speed and the reproducibility in the hands of many workers are higher with the avidin-biotin mediated linkage than with the chemical linkage.

It is important to emphasize that the labeling efficiencies achieved here allow analysis only of defined segments of DNA. Specifically, this means that a fixed point or marker is needed from which to map the labeled genes. Such a reference point could be a restriction endonuclease cleavage site, a substitution or deletion loop in a heteroduplex, a secondary structure feature in the DNA or a long duplex region formed by hybridizing specific RNA or DNA probes (8, 40). When such systems are studied with this technique, it is possible in a single experiment to obtain a gene map with a resolution of several hundred nucleotides.

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